Pichia garciniae sp. nov., isolated from a rotten mangosteen fruit (Garcinia mangostana L., Clusiaceae)

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Yeasts of the genera Rhodotorula, Cryptococcus, Sporobolomyces, Saccharomyces, Candida and Pichia, amongst others, have been isolated from fresh and rotten fruits (Fleet, 2003; Rao et al., 2007; Bhadra et al., 2007; Slavikova et al., 2007). These fruit-associated yeasts produce extracellular enzymes such as lipases, cutinases and pectinases and thus hasten the spoilage of fruits during storage and transportation (Fleet, 2003). A number of yeasts in the decaying mangosteen fruit (Garcinia mangostana L., Clusiaceae), a number of other yeasts have been isolated. During the course of a study to identify yeasts associated with a decaying mangosteen fruit (Garcinia mangostana L., Clusiaceae), a number of yeast strains were isolated. Detailed polyphasic taxonomic analysis indicated that the strains are representatives of the genus Pichia. Based on biochemical characteristics and the sequence of the D1/D2 domain of the 26S rRNA gene and the ITS1–5.8S rRNA gene–ITS2 region, strains YS110T and YS111 represent a novel species of the genus Pichia.

Mangosteen fruits (about 1 kg) were purchased in May 2007 from a market in the city of Bangkok, Thailand, and transported to Hyderabad, Andhra Pradesh, India. Most of the fruits remained unspoiled for about a week after one of them showed visible signs of rotting. A small piece from the rotten fruit (approx. 1 g) was sliced using a sterile scalpel blade and suspended in 2 ml sterile distilled water by vigorous vortexing. The resulting suspension (100 µl) was then plated on YM agar plates (l-1: 5 g peptone, 3 g yeast extract, 3 g malt extract, 10 g glucose and 15 g agar) supplemented with 50 µg chloramphenicol ml−1 and the plates were incubated at 28 °C for 48 h. The abundance of yeasts in the decaying mangosteen fruit was 2.0–2.2×10² c.f.u. g−1. A total of 32 yeast colonies appeared on YM-chloramphenicol plates, which could be grouped into four morphotypes based on the colour and shape of the colonies. Three representatives of each colony morphotype were purified by repeated streaking on YM agar plates.

The GenBank/EMBL/DDBJ accession numbers for the 18S rRNA gene (partial)–ITS1–5.8S rRNA gene–ITS2–26S rRNA gene (partial) sequences of strains YS110T and YS111 are respectively AM882676–AM882678, and those of the D1/D2 domains of the 26S rRNA genes of strains YS119, YS118 and YS117 are AM882676–AM882678, respectively.

RAPD profiles from 12 novel yeast isolates are available as supplementary material with the online version of this paper.
without the antibiotic and genomic DNA of the 12 isolates was extracted (Bhadra et al., 2007) and purified (Makimura et al., 1994). Following RAPD analysis using (GTG)3 as a primer (Bhadra et al., 2007), the 12 isolates could also be classified into four groups, G-I, G-II, G-III and G-IV, based on the banding pattern of the PCR products when resolved on a 1 % agarose gel (Supplementary Fig. S1, available in IJSEM Online). One or two representatives from each group, YS110T and YS111 from G-I, YS117 from G-II, YS118 from G-III and YS119 from G-IV, were then subjected to detailed phylogenetic analysis.

For routine subculturing and maintenance, the strains were grown on/in YM agar/broth at 28 °C. Morphological, physiological and biochemical properties were determined as described by Yarrow (1998). All assimilation tests were performed twice and results were scored after both 1 and 3 weeks. An Axioplan microscope (Zeiss) was used to visualize the morphology of the vegetative cells and ascospores. Strains were grown on potato-dextrose-rose bengal agar (M938; HiMEDIA) (1: 1 g glucose, 0.008 g rose bengal, 0.05 g chloramphenicol and 20 g agar) and Gorodkowa agar (1: 1 g glucose, 10 g peptone, 5 g NaCl and 18 g agar) and visualized after 3, 5, 7, 10 and 12 days for ascospore formation using a phase-contrast microscope under a ×100 oil-immersion objective.

_Candida albicans_ JCM 1542T, _P. membranifaciens_ NRRL Y-2026T and _P. manshurica_ NRRL Y-17978T were used as reference strains for phenotypic studies.

Quinones were extracted and purified from cells grown in 250 ml YM broth for 3–5 days according to Yamada et al. (1989). The dark band of Co-Q observed under UV light was scraped and extracted with 2 ml acetone and identified using reversed-phase HPTLC (Thanh et al., 2003) using Co-Q standards (Sigma-Aldrich). _Saccharomyces cerevisiae_ NRRL Y-12632T, _Pichia fermentans_ NRRL Y-1619T, _P. membranifaciens_ NRRL Y-2026T, _Debaryomyces hansenii_ NRRL Y-17941T and _Schizosaccharomyces pombe_ NRRL Y-12796T were used as reference strains for Co-Q analysis. The G+C content of the DNA was determined from melting point (Tm) curves (Sly et al., 1986) obtained using a Lambda 2 UV-visible spectrophotometer (Perkin Elmer) equipped with Templab 2.0 software package (Perkin Elmer). DNA–DNA hybridization was performed using a DIG DNA labelling and detection kit (Roche Diagnostics) according to Bhadra et al. (2005).

The internal transcribed spacer (ITS) region including the 5.8S rRNA gene (ITS1–5.8S rRNA gene–ITS2) and the D1/D2 domain of 26S rRNA gene of isolates YS110T, YS111, YS117, YS118 and YS119 were amplified using the primers ITS1 and NL4 and the amplicon was sequenced using the primers NL1, NL2A, NL3A, ITS3 and ITS4 (Lin et al., 1995; Kurtzman & Robnett, 1998). Details of amplification and sequencing protocols are given elsewhere (Bhadra et al., 2007). Sequences were corrected manually and aligned using CLUSTAL_X (Thompson et al., 1997). Pairwise alignment studies were done using the ExPASy Bioinformatics online software tools (http://www.expasy.ch/tools/sim-prot.html). Phylogenetic analyses were conducted using MEGA 3.1 (Kumar et al., 2004) according to Rao et al. (2007). Bootstrap analysis (Felsenstein, 1985) was performed for 1000 replications. Reference sequences were retrieved from GenBank under the accession numbers indicated in the tree (Fig. 1).

Pairwise alignment analysis of the nucleotide sequences of the D1/D2 domains of the 26S rRNA gene of strains YS117, YS118 and YS119 showed 100 % similarity to those of _Pichia anomala_ NRRL Y-366T (GenBank accession no. U74592; not shown in Fig. 1), _P. fermentans_ NRRL Y-1619T and _P. manshurica_ NRRL Y-17349 (the type strain of _Pichia galeiformis_), respectively. In the neighbour-joining phylogenetic tree constructed using D1/D2 sequences, YS117 grouped with _P. anomala_ NRRL Y-366T (data not shown), whereas YS118 and YS119 grouped with _P. fermentans_ NRRL Y-1619T and _P. manshurica_ IFO 10726T, respectively (Fig. 1). These strains also showed similar biochemical characteristics to their nearest phylogenetic relatives and produced ascospores after 5 days of incubation on potato-dextrose-rose bengal agar plates (not shown). Therefore, strains YS117, YS118 and YS119 are assumed to be strains of _P. anomala_, _P. manshurica_ and _P. fermentans_, respectively.

The nucleotide sequences of the D1/D2 domain of the 26S rRNA gene and the ITS1–5.8S–ITS2 region of representative strains of G-I, YS110T and YS111, were almost identical (99.9 % similarity). BLAST analysis of the nucleotide sequence of the D1/D2 domain of YS110T indicated that the nearest phylogenetic neighbours are _P. membranifaciens_ and _P. manshurica_. Strain YS110T showed 97 % similarity with _P. manshurica_ IFO 10726T, 96.3 % similarity with _P. membranifaciens_ NRRL Y-2026T, 96.1 % similarity with _Pichia deserticola_ NRRL Y-12918T and 96 % similarity with _Candida ethanolica_ NRRL Y-12615T. The phylogenetic tree constructed using the neighbour-joining method based on D1/D2 sequences further strengthens the conclusion that YS110T is most closely related to _P. membranifaciens_, with a bootstrap support of 99 % (Fig. 1). The nucleotide sequence of the ITS1–5.8S rRNA gene–ITS2 region of strain YS110T shows 85.1 % similarity to that of _P. membranifaciens_ CBS 209 (GenBank accession no. DQ104714) and 81.4 % similarity to that of _P. manshurica_ IFO 10726T (AB054036). Based on rRNA gene sequencing and electrophoretic karyotyping, Wu et al. (2006) reported that strains identified as _P. membranifaciens_ could show 5–13 % variation in their ITS1–5.8S rRNA gene–ITS2 region nucleotide sequence. Therefore, a DNA–DNA relatedness study was conducted between strains YS110T and YS111, _P. membranifaciens_ NRRL Y-2026T and _P. manshurica_ NRRL Y-27978T. Strains YS110T and YS111 showed 92 % DNA–DNA relatedness with each other and both strains showed 52–57 % DNA–DNA relatedness with _P. membranifaciens_ NRRL Y-2026T and _P. manshurica_ NRRL Y-27978T.

Previous studies have shown that strains with >1 % substitution in the D1/D2 domain sequence usually
represent separate species (Kurtzman & Robnett, 1997, 1998; Lu et al., 2004; Suh & Blackwell, 2004). Strains YS110T and YS111 differ by 3–3.7 % in their D1/D2 sequence and 14.9–18.6 % in their ITS1–5.8S rRNA gene–ITS2 region sequence from the type strains of P. membranifaciens and P. manshurica. The DNA–DNA relatedness of YS110T and YS111 with the type strains of P. membranifaciens and P. manshurica was 70 %. Therefore, based on phylogenetic analysis and DNA–DNA relatedness studies, strains YS110T and YS111 should be regarded as strains of a novel species of the genus Pichia. Strains YS110T and YS111 do not differ significantly in their phenotypic properties from strains of P. membranifaciens as reported by Barnett et al. (2000) but, when YS110T and YS111 were compared directly in this study with the type strain of P. membranifaciens, NRRL Y-2026T, differences were clearly visible (Table 1). The strains produce two to four ascospores after 3–5 days of incubation on potato-dextrose-rose bengal agar and Gorodkowa agar (Fig. 2). Thus, based on phenotypic differences, phylogenetic analysis and DNA–DNA relatedness studies, strains YS110T and YS111 represent a novel species of Pichia, for which the name Pichia garciniae sp. nov. is proposed.

Latin diagnosis of Pichia garciniae Bhadra et Shivaji sp. nov.

Table 1. Phenotypic characteristics of strains YS110T and YS111, *P. membranifaciens* and *P. manshurica*

Data were obtained in this study unless indicated. All strains assimilate D-glucose but not D-galactose, L-sorbose, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, sucrose, maltose, cellobiose, salicin, melibiose, lactose, raffinose, melezitose, starch, erythritol, ribitol, *myo*-inositol, D-glucurate, D-glucuronate, propane-1,2-diol or butane-2,3-diol as sole carbon sources. All strains assimilate ethylamine, L-lysine, cadaverine and glucosamine but not nitrate, nitrite, creatinine or imidazole as sole nitrogen sources. All strains are sensitive to 0.1 % cycloheximide and do not grow in medium containing 1 % (v/v) methanol. None of the strains form starch-like compounds or hydrolyse urea and all strains give a negative diazonium blue B reaction. +, Positive; w, weakly positive, D, variable; –, negative.

<table>
<thead>
<tr>
<th>Property</th>
<th>Strains YS110T and YS111</th>
<th><em>P. membranifaciens</em></th>
<th><em>P. manshurica</em> NRRL Y-27978T</th>
<th>Barnett et al. (2000)</th>
</tr>
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<tr>
<td>Fermentation of D-glucose</td>
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<td>D</td>
<td>D</td>
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<td>Assimilation of:</td>
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<td>D-Glucosamine</td>
<td>W</td>
<td>W</td>
<td>+, –</td>
<td>–</td>
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<tr>
<td>Inulin</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>W</td>
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<td>Glycerol</td>
<td>+</td>
<td>+</td>
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<td>Xylitol</td>
<td>+</td>
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<td>Succinate</td>
<td>+</td>
<td>D</td>
<td>+, –</td>
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<td>Citrate</td>
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<td>Growth in:</td>
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<tr>
<td>Acetic acid (1 %)</td>
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<td>–</td>
<td>+, –</td>
<td>–</td>
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<tr>
<td>Cycloheximide (0.01 %)</td>
<td>+</td>
<td>–</td>
<td>D</td>
<td>–</td>
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<td>D-Glucose (50 %)</td>
<td>–</td>
<td>W</td>
<td>+, –</td>
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</table>

**Description of *Pichia garciniae* Bhadra & Shivaji sp. nov.**

*Pichia garciniae* (gar.ci’ni.ae. N.L. gen. fem. n. *garciniae* of *Garcinia*, a botanical genus name, referring to the isolation of the type strain from rotting fruit of mangosteen, *Garcinia mangostana* L.).

After 3 days on YM agar at 28 °C, cells are spherical or ovoid (4–7 × 3.5–4 μm) and occur singly, in pairs or in groups (Fig. 2). Budding is multilateral. After 1 month at 28 °C, sediment and pellicle formation is observed. On YM agar medium after 15 days at 28 °C, the culture is butyrous, pale brownish in colour, smooth with a rough margin. Pseudohypha formation is not observed on YM agar or cornmeal agar plates after 10–21 days of incubation at 28 °C. After 3–5 days of incubation at 28 °C on Gorodkowa agar and potato-dextrose-rose bengal agar, conjugation is followed by formation of two to four hat-shaped ascospores per cell. Ascii deliquescent. Cells do not ferment D-glucose. Assimilates D-glucose, D-glucosamine (weakly), glycerol, xylitol and succinate but does not assimilate D-galactose, L-sorbose, D-ribose, D-xylose, L- or D-arabinose, L-rhamnose, sucrose, maltose, cellobiose, salicin, melibiose, lactose, raffinose, melezitose, starch, erythritol, ribitol, *myo*-inositol, D-glucurate, D-glucuronate, citrate, propane-1,2-diol or butane-2,3-diol as sole carbon sources. Assimilates ethylamine, L-lysine, cadaverine and glucosamine but does not assimilate nitrate, nitrite, creatinine or imidazole as sole nitrogen sources. Does not grow in vitamin-free media. Strains are sensitive to 0.1 % cycloheximide and do not grow in medium containing 1 % (v/v) acetic acid or methanol. Resistant to 0.01 % cycloheximide and does not grow in 50 % glucose. Does not form starch-like compounds and does not hydrolyse urea and the diazonium blue B reaction is negative.
Coenzyme Q7 is the major ubiquinone. The G+C content of the DNA of the type strain is 48.5 mol%.

The type strain is YS110T (=NRRL Y-48422T =CBS 10758T), isolated from a decaying mangosteen fruit (Garcinia mangostana L., Clusiaceae).

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References


